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13. ABSTRACT (Maximum 200 Words)

Epidemiological and laboratory studies show that boron, selenium and zinc reduce prostate cancer risk whereas calcium and cadmium increase risk. The objective of this proposal is to determine the concentration and location of these elements in normal and tumor tissue. Specific aims include: (1) preparation of four grades of prostate tissue, (2) determination of tissue concentrations of: B, Ca, Cd, Se and Zn; and (3) determination of tissue and cellular distribution of these elements using a NanoSIMS ion microscope at Lawrence Livermore National Laboratory (LLNL). Progress toward specific aim 1 includes preparing matched samples of normal and graded tumor tissues at prostatectomy. Progress toward specific aim 2 includes the ICPMS analysis of 23 paired tissue samples of normal and Gleason graded tumor tissue. The association between Gleason scores and elemental concentrations were not statistically significant. Work toward specific aim 3 includes the development of slam freezing and freeze substitution procedures. Training was completed on the NanoSIMS ion microscopy at LLNL and sample analysis was begun. Preliminary NanoSIMS results show that each element has a unique cellular distribution pattern with some overlap for example B and Ca. During the next project year the compartmentalization both within cells and within prostate tissue will be determined for different Gleason grades of prostate tissue.

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INTRODUCTION

There is growing evidence that the elements boron, selenium and zinc reduce PCa risk whereas calcium and cadmium increase risk (1-14). The objective of this proposal is to determine if these elements differ in concentration and location between normal and tumor tissue. During the first year of the project pathologically graded human prostate tissue was analyzed to determine the concentrations of boron, calcium, cadmium, selenium and zinc. There was no statistical association between gross tissue concentrations and Gleason score. The major goals for the second year were to receive training on use of the NanoSIMS microscopy at Lawrence Livermore National Laboratory (LLNL), develop sample preparation procedures for the analysis of tissue on the instrument, and begin analysis. These goals were accomplished and early analysis is showing that each element has a unique cellular distribution pattern. During the third project year we will concentrate on characterizing the compartmentalization of the elements both within cells and within prostate tissue of different Gleason grades. It is expected that elemental location, rather than concentration, will provide insight into how the elements under study are able to modify PCa risk.

BODY

Task 1. To identify and maintain a series of progressively dedifferentiated samples (months 1-30).

a. Obtain specimens from radical prostatectomy from the UCLA Human Tissue Research Center.

Accomplishments: Unmatched and matched sets of normal and tumor prostate tissue were collected from the UCLA Human Tissue Research Center. Unmatched samples were used to establish the methods for elemental analysis.

b. Develop four (donor/tissue) classification categories that reflect increased risk of prostate cancer (normal man/normal tissue; cancer patient/very mild dysplasia (Gleason's grade total 2-4), cancer patient/moderately differentiated adenocarcinoma (Gleason's grade total = 5-7) and poorly differentiated adenocarcinoma (Gleason's grade total = 8-10).

Accomplishments: We used normal tissue from men with cancer because samples of normal tissue from normal men are nearly non-existent. The vast majority of tissue samples available have Gleason scores in the range from 6 to 7. Sufficient samples were obtained during the first year to accomplish Task 2. We continue to obtain tissues in all categories for elemental analysis.

Task 2. To utilize state of the art inductive coupled plasma mass spectrometry to determine the concentration of the B,Ca, Cd, Se and Zn in whole tissue samples (Months 1-12).

a. Analyze the four classification categories of biopsy tissue by inductively coupled plasma mass spectrometry (ICPMS) to obtain whole tissue concentrations of: B, Ca, Cd, Se and Zn.

Accomplishments: We analyzed matched pairs of normal and tumor tissue to determine the concentrations of B, Ca, Cd, Se and Zn in 23 different male donors. The mean, median, range and coefficient of variation of the concentrations are given in Table 1.

Table 1. Elemental Concentrations of Normal and Tumor Prostate Tissue in 23 Men

Normal		Boron	Cadmium	Calcium	Selenium	Zinc
		ng/g	ng/g	µg/g	ng/g	µg/g
	Mean	291	71	313	471	86
	Median	90	58	300	480	73
	Range	28 - 3530	8 - 220	82 - 830	200 - 730	9 - 190
	Std Error	159	10	36	24	11
	CV	256%	66%	56%	25%	63%
Tumor						
	Mean	355	83	263	502	88
	Median	100	61	270	520	84
	Range	23 - 2360	20 - 250	80 - 430	15 - 820	110-180
	Std Error	129	12	18	34	9
	CV	170%	70%	33%	33%	50%

The concentrations in both normal and tumor tissue ranked as follows: Ca > Zn > Se > B > Cd. A statistical comparison of elemental concentrations in normal and tumor tissue did not reveal significant differences (Table 2). The coefficient of variation of the elements varied greatly between elements (Table 1). The magnitude of variation followed the same rank in normal and tumor tissue: B > Cd > Zn > Ca > Se. The high variation in boron concentrations was not expected. Boron is not known to activate or covalently bind to proteins. It's variability and 10 fold range in concentration suggests that prostate is able to accumulate boron. Examination of tissue using the NanoSIMS ion probe will determine where this occurs for boron and the other elements.

Table 2. Statistical Evaluation of Elemental Concentrations in Matched Normal and Tumor Tissue	
Element	Statistical Comparison between Normal and Tumor Tissue
Boron[1]	$p = 0.31$
Calcium[1]	$p = 0.46$
Cadmium[1]	$p = 0.58$
Selenium[1]	$p = 0.21$
Zinc[2]	$p < 0.09$

b. Determine the strength of the relationship between whole tissue elemental concentrations and pathological tissue classification by statistical analysis.

Accomplishments: Table 3 shows there was no relationship between Gleason score and the concentration of any element.

Table 3. Statistical Evaluation of the Relationship between Gleason Scores and Elemental Concentrations in Tumor Tissue	
Element	Correlation Coefficient of Gleason Score versus Element Concentration
Boron[1]	$R = 0.10$
Calcium[1]	$R = 0.13$
Cadmium[1]	$R = 0.08$
Selenium[1]	$R = 0.30$
Zinc[2]	$R = 0.21$

Each of these elements had been positively or negatively associated with prostate cancer risk, but this did not show up as concentration differences at the gross tissue level. NanoSIMS analysis will provide information on how the elements are distributed within prostate tissue. This information is needed to provide insight into the basis of the biological variability.

Task 3. To determine the microlocation and microconcentrations of B, Ca, Cd, Se and Zn in graded series of samples (Months 6-36).

a. Ion and photographic imaging of the four pathological categories of biopsy tissues will be accomplished using the ion microscope (NanoSIMS 50) located in the Analytical and Nuclear Chemistry Division of the Lawrence Livermore National Laboratory.

Accomplishments: Mapping elemental locations in cells and tissue requires that the integrity of fine structure be maintained. Ice crystals form in tissues frozen at -80°C and above and this causes membrane rupture and movement of soluble ions, molecules and even granules and vesicles. When tissues are rapidly frozen in liquid nitrogen the outer 20 microns reaches temperatures less than -80°C before crystal formation, but at greater

tissue depths crystals form and disrupt the fine structure. During year 2 we employed slam freezing to prevent crystal formation in outer layers of prostate tissues and then focused on the outer layers in our NanoSIMS analysis. Ice crystals also form if water is not removed before the tissue is returned to temperatures above -80°C. To prevent this we used a freeze substitution method to replace water with acetone before the sample was warmed above -80°C. In year three we will switch to a procedure used by our LLNL colleagues. They use a Turbo Freeze Drier that slowly sublimates water as tissue temperature is increased from -140°C to - 10°C under vacuum. This procedure does not require acetone and preserves fine structure. The use of the Turbo Freeze Drier during year three will assure the natural structure of our Gleason graded tissue is maintained for NanoSIMS analysis.

Our LLNL colleagues recommended that we prepare cultured cells for training and initial analysis on the NanoSIMS microscope. Their request was an opportunity for us to control the concentration of the most difficult element to measure, boron. The results assured us that the NanoSIMS was sufficiently sensitive to pick up a boron signal. Below is a picture of a prostate cancer cell (DU-145) analyzed using the NanoSIMS microscope showing light areas of carbon nitrogen polyatomic atoms.

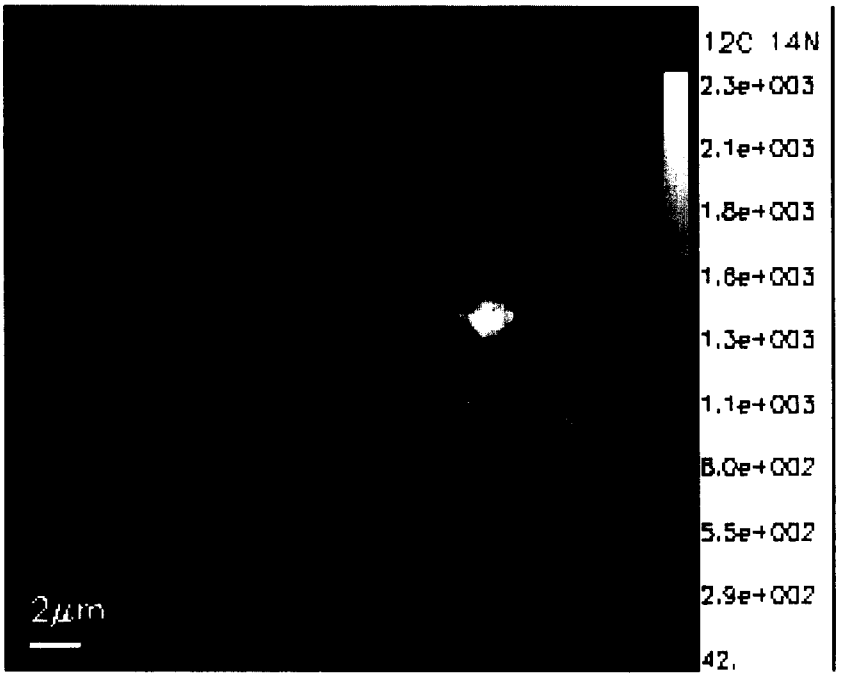

Figure 1. The light areas represent the origin of secondary carbon-nitrogen polyatomic ions sputtered from the sample by primary cesium positive ions rastered across the cells. The C-N ion pairs are at their highest levels around the nucleus and within the nucleolus. The numbers to the right of the image indicate C-N ion pairs emitted per carbon atom from the same region.

b. Ion and photographic images will be analyzed using image analysis software to locate cellular sites of elemental accumulations. An example of this is the cellular distribution of the Ca, B and Na atoms shown in Figure 2.

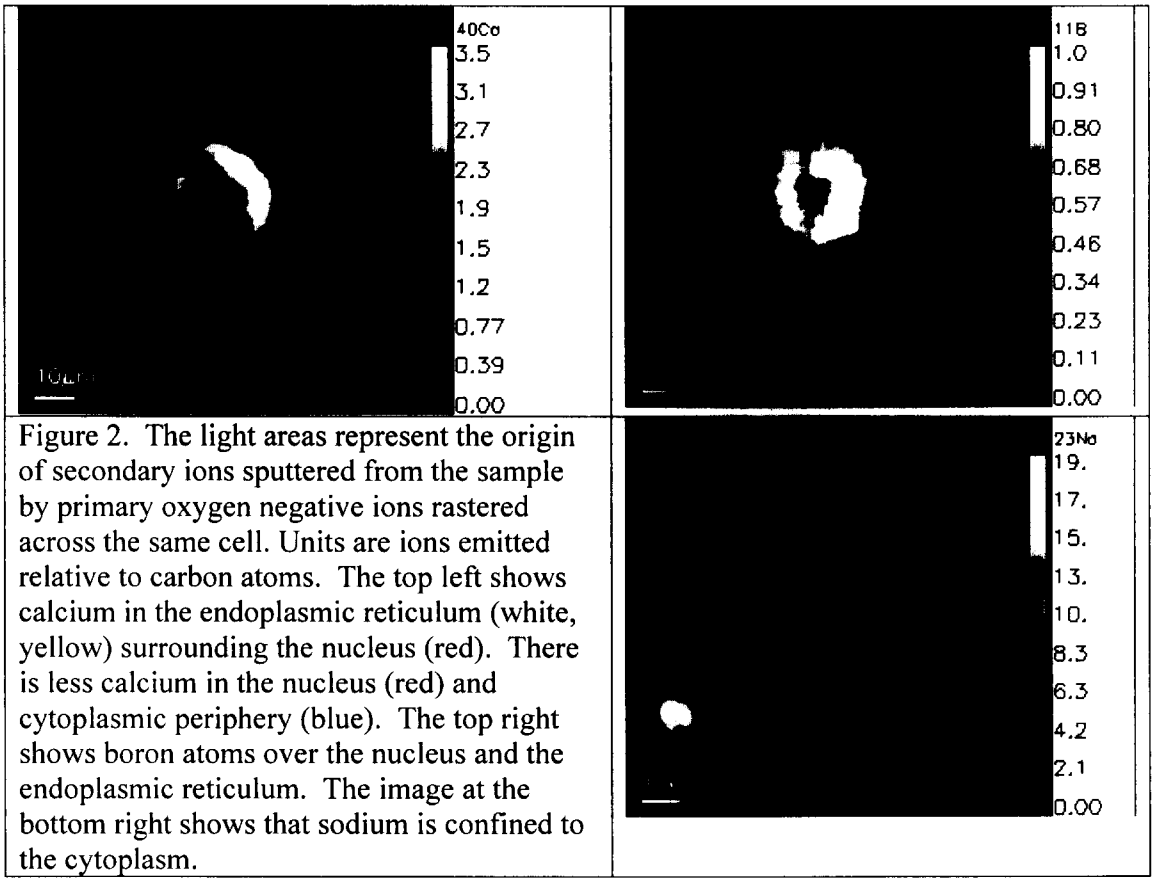

Figure 2. The light areas represent the origin of secondary ions sputtered from the sample by primary oxygen negative ions rastered across the same cell. Units are ions emitted relative to carbon atoms. The top left shows calcium in the endoplasmic reticulum (white, yellow) surrounding the nucleus (red). There is less calcium in the nucleus (red) and cytoplasmic periphery (blue). The top right shows boron atoms over the nucleus and the endoplasmic reticulum. The image at the bottom right shows that sodium is confined to the cytoplasm.

c. The relative concentrations of the elements at concentrated sites will be obtained in relationship to K and Na from the ion and photographic images using image analysis software.

This goal has been modified. The NanoSIMS software uses a relative concentration per carbon atom. The K and Na values will be used to assure membrane integrity

d. The spatial distribution and concentrations of elements will be used to determine if statistically meaningful correlations exist between the concentration of elements at specific cellular targets and the pathological classification of cancer.

This is a goal for year three. The ions for a maximum of five different elements can be quantitated over a single image. In project year 3, the cellular location and relative concentration for each of the elements will be obtained and used for this analysis.

e. Graphic representations will be used to show the relationship between concentrations in whole tissue as well as microcellular deposits of the elements, and the differentiation state of the tissue.

This is a goal for year three.

Accomplishments: Progress on Task 3 focused on the development of methods to prepare tissue for ion microscopy. We purchased a Leica EM MM80 slam freezer to ultra-rapidly freeze prostate tissue and a Thermo Neslab CC-100 cold probe to regulate the dehydration phase. Briefly, the Leica slam freezer was prechilled by filling with liquid nitrogen. The tissue was slam frozen and transferred to a Dewar containing acetone chilled to – 83°C. The temperature of the acetone bath was controlled using the Thermo Neslab CC-100 probe and refrigeration unit. Samples were slowly brought to room temperature over a period of 72 hours. The dehydrated tissue was fixed in osmium and embedded in Spurr Low Viscosity Resin.

This procedure was successful for cultured prostate cells used in training, however, our colleagues at Lawrence Livermore recommend using a Turbo Freeze Drier for freeze substitution. We plan to modify our procedure according to their recommendations and purchase a Turbo Freeze Drier for use in year three. This will require using about $30,000 of the budget to purchase this instrument.

Potential Problem Scheduling NanoSIMS Time

The NanoSIMS microscope at LLNL is a multimillion dollar instrument that is complicated to run and maintain. Although it is only one year old and has full time personnel to keep it operational, it requires down time for repairs that could slow our progress. In addition, national security agencies have a periodic need for sample analysis on the NanoSIMS. If our colleagues at LLNL need to run samples for the FBI or other national security agencies this could cause delay. On the positive side, there a national security need in biodefense to train people who can use this highly sophisticated instrument and my students are being trained to fill that need and this gives us some priority.

KEY RESEARCH ACCOMPLISHMENTS

- Completion of Task 1: Successful procurement of Gleason graded tissue classified as normal, Gleason grades 3, 5, 6, 7, 8.
- Completions of Task 2: Analysis of matched sets of normal and tumor tissue obtained from 23 different men for the elements, boron, calcium, cadmium, selenium and zinc.
- Statistical analysis of elemental values of the matched sets
- Development of procedures required to prepare samples for NanoSIMS analysis
- Training on the use of the NanoSIMS microscope at Lawrence Livermore National Laboratories.
- Initiation of NanoSIMS analysis of prostate cells and tissues

REPORTABLE OUTCOME

Work supported by this grant resulted in a presentation (abstract) at the 2004 Experimental Biology Meetings and a peer reviewed publication in *Cancer Letters*. The work for the *Cancer Letters* paper provided cells of increasing concentration of boron, the most difficult to measure element on the NanoSIMS.

Eckhert, C.D. Concentration and variation of boron, selenium and elements associated with cancer risk in non-tumor human prostate tissue. *FASEB J.* 2004; 18:A351.3 (351.3).

Barranco W.T. and Eckhert C.D. Boric acid inhibits human prostate cancer cell proliferation. *Cancer Letters* 216:21-29, 2004.

The project provided training and support for three students and the PI. The following list of people received pay from the research effort.

Kim Henderson (Student)
Joey Miller (Student)
Wade Barranco (Student)
Curtis Eckhert (PI)

CONCLUSIONS

The project is on schedule. Task 1 and 2 were accomplished during year one. This consisted of the collection and analysis of 23 matched pairs of normal/tumor prostate tissue. During the second year an abstract was presented at the Experimental Biology Meetings, a paper published in *Cancer Letters* as a part of completing Task 3. Training was completed at the Lawrence Livermore National Laboratory on use of the NanoSIMS ion microscope. The procedures for tissue preparation were developed for NanoSIMS analysis. Prostate tumor cells cultured in different concentration of boron, the most difficult element to measure, were used to demonstrate the ability of the NanoSIMS microscope to discriminate concentration differences. It has been recommended that we purchase a Turbo Freeze Drier for freeze substitution of tissue samples. We will use currently funds from project year three to do this. Work during the third project year will concentrate on determining the compartmentalization of the elements both within cells and within prostate tissue for different Gleason grades of prostate tissue.

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